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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>A61K 31/00, 31/70, 31/16, 31/365, 31/135, 31/19, 31/555, 31/66</b>		<b>A3</b>	(11) International Publication Number: <b>WO 98/29108</b> (43) International Publication Date: <b>9 July 1998 (09.07.98)</b>
(21) International Application Number: <b>PCT/US97/23991</b> (22) International Filing Date: <b>29 December 1997 (29.12.97)</b> (30) Priority Data: <b>60/034,095 30 December 1996 (30.12.96) US</b> (63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application <b>US 60/034,095 (CON)</b> <b>Filed on 30 December 1996 (30.12.96)</b> (71)(72) Applicant and Inventor: <b>FEINBERG, Andrew, P.</b> <b>[US/US]; 15 Westspring Way, Lutherville, MD 21093</b> <b>(US).</b> (74) Agents: <b>SPRUILL, W., Murray et al.; Bell Seltzer Intellectual</b> <b>Property Law Group, Alston &amp; Bird LLP, P.O. Drawer</b> <b>34009, Charlotte, NC 28234 (US).</b>			(81) Designated States: <b>AL, AM, AT, AT (Utility model), AU, AZ,</b> <b>BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, CZ (Utility</b> <b>model), DE, DE (Utility model), DK, DK (Utility model),</b> <b>EE, EE (Utility model), ES, FI, FI (Utility model), GB, GE,</b> <b>GH, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK,</b> <b>LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO,</b> <b>NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility</b> <b>model), SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU,</b> <b>ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG,</b> <b>ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ,</b> <b>TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR,</b> <b>GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF,</b> <b>BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</b>  <b>Published</b> <i>With international search report.</i> (88) Date of publication of the international search report: <b>18 February 1999 (18.02.99)</b>

(54) Title: **COMPOSITIONS AND METHODS FOR RESTORING A NORMAL PATTERN OF IMPRINTING TO CELLS**

(57) Abstract

Compositions and methods for the diagnosis and treatment of diseases that are associated with imprinting are provided. Such compositions and methods are useful in restoring a normal pattern of imprinting to cells. The compositions comprise pharmacological agents that are involved in imprinting and can be used in preventive and therapeutic methods to restore a normal pattern of imprinting. Such agents are capable of restoring a normal pattern of imprinting to cells. Further methods of the invention include means for identifying pharmacological compounds that exert their effect by restoring normal imprinting to abnormally imprinted chromosomes and genes. Additionally, the invention could also be used to identify new compounds that exert their effect by restoration of normal imprinting to diseased tissues that exhibit underlying abnormal imprinting.

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## INTERNATIONAL SEARCH REPORT

 International Application No  
 PCT/US 97/23991

## A. CLASSIFICATION OF SUBJECT MATTER

 IPC 6 A61K31/00 A61K31/70 A61K31/16 A61K31/365 A61K31/135  
 A61K31/19 A61K31/555 A61K31/66

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
L	J.M. BARLETTA ET AL.: "Reversal of Loss of Imprinting in Tumor Cells by 5-Aza-2'-deoxycytidine" HTTP://AACR.EDOC.COM/CR/V57N1/CH000048.HTM L, XP002066097 see abstract	1-13, 17-19
L	& "Cancer Research; Instructions for Authors" HTTP://WWW.AACR.ORG/CANINST.HTML, pages 1-19, Guidelines B-VI, 5.3; B-X, 9.2, (viii) see page 2	1-13, 17-19
T	& J.M.BARLETTA ET AL.: "Reversal of Loss of Imprinting in Tumor Cells by 5-Aza-2'-deoxycytidine" CANCER RESEARCH, vol. 57, no. 1, 1 January 1997, pages 48-50, see the whole document -/--	1-13, 17-19

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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\*Z\* document member of the same patent family

Date of the actual completion of the international search

3 July 1998

Date of mailing of the international search report

30. 11. 98

Name and mailing address of the ISA

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# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 97/23991

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>--- P. EVERSOLE-CIRE ET AL.: "Activation of an Imprinted Igf 2 Gene in Mouse Somatic Cell Cultures" MOL.CELL.BIOL., vol. 13, no. 8, August 1993, pages 4928-4838, XP002066098 see the whole document</p>	1-13, 17-19
X	<p>--- HTTP://CPMCNET.COLUMBIA.EDU/NEWS/JOURNAL/ARCHIVES/JOUR_V15N1_0005.HTML, XP002070304 &amp; "Wilms' Tumor Gene Research" P&amp;S JOURNAL, vol. 15, no. 1, 1995, see the whole document</p>	1-13, 17-19 1-13, 17-19
X	<p>--- WO 92 15602 A (DANA-FARBER CANCER INSTITUTE, INC.) 17 September 1992 see page 25 - page 26; claims 6,12,13</p>	1-13, 17-19
X	<p>--- J. WEBER ET AL.: "Expression of the MAGE-1 Tumor Antigen Is Up-Regulated by the Demethylating Agent 5-Aza-2'-Deoxycytidine" CANCER RES., vol. 54, no. 7, 1 April 1994, pages 1766-1771, XP002066099 see the whole document</p>	1-13, 17-19
X	<p>--- S.W.LEE ET AL.: "Down-regulation of a member of the S100 gene family in mammary carcinoma cells and reexpression by azadeoxycytidine treatment" PROC.NATL.ACAD.SCI. USA, vol. 89, no. 6, March 1992, pages 2504-2508, XP002066100 see the whole document</p>	1-13, 17-19
X	<p>--- A.T.FERGUSON ET AL.: "Demethylation of the Estrogen Receptor Gene in Estrogen Receptor-negative Breast Cancer Cells Can Reactivate Estrogen Receptor Gene Expression" CANCER RES., vol. 55, no. 11, 1 June 1995, pages 2279-2283, XP002066101 see the whole document</p> <p>---</p>	1-13, 17-19

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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 97/23991

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>M.CARAGLIA ET AL.:            "5-Aza-2'-deoxycytidine induces growth inhibition and upregulation of epidermal growth factor receptor on human epithelial cancer cells"            ANN.ONCOL.,            vol. 5, no. 3, March 1994, pages 269-276,            XP002066102            see the whole document</p>	1-13, 17-19
X	<p>W.-Y. CHUNG ET AL.: "Chromosome 11p15.5 regional imprinting: comparative analysis of KIP2 and H19 in human tissues and Wilm's tumors"            HUM.MOL.GENET.,            vol. 5, no. 8, August 1996, pages 1101-1108, XP002066103            see the whole document</p>	1-13, 17-19
X,P	<p>B. TYCKO: "DNA methylation in genomic imprinting"            MUTATION RESEARCH,            vol. 386, no. 2, 1997, pages 131-140,            XP002070305            see abstract</p>	1-13, 17-19
A	<p>L.A.MICHALOWSKY ET AL.: "DNA Methylation and Differentiation"            ENVIRON.HEALTH PERSPECT.,            vol. 80, March 1989, pages 189-197,            XP002066104            see abstract</p>	1
X	<p>J.-F. HU ET AL.: "Promotor-specific Modulation of Insulin-like Growth Factor II Genomic Imprinting by Inhibitors of DNA Methylation"            J.BIOL.CHEM.,            vol. 271, no. 30, 26 July 1996, pages 18253-18262, XP002066105            see the whole document</p>	1-13, 17-19
A	<p>A.H.BEGGS ET AL.: "Reactivation of X-Linked Genes in Human Fibroblasts Transformed by Origin-Defective SV40"            SOMAT.CELL MOL. GENET.,            vol. 12, no. 6, November 1986, pages 585-594, XP002066106            see abstract</p>	1
	<p>---</p> <p>-/--</p>	

# INTERNATIONAL SEARCH REPORT

International Application No

PC 1/US 97/23991

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	S.M. TAYLOR: "5-Aza-2'-Deoxycytidine: Cell Differentiation and DNA Methylation" LEUKEMIA, vol. 7, no. Suppl. 1, May 1993, pages 3-8, XP002066107 see the whole document	1-13, 17-19
A	S. RAINIER ET AL.: "Capture and characterization of 5-aza-2'-deoxycytidine-treated C3H/10T1/2 cells prior to transformation" PROC.NATL.ACAD.SCI. USA, vol. 85, no. 17, September 1988, pages 6384-6388, XP002066108 see abstract	1
A	EP 0 627 436 A (TSUMURA & CO.) 7 December 1994 see claims 1-4	1
A	US 4 497 803 A (HARADA ET AL.) 5 February 1985 see column 2, line 22 - line 28	1
A	US 5 356 903 A (EISSENSTAT ET AL.) 18 October 1994 see column 16, line 54 - column 17, line 23	1
A	US 4 690 918 A (BEPPU ET AL.) 1 September 1987 see the whole document	1

# INTERNATIONAL SEARCH REPORT

national application No.

PCT/US 97/23991

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
see FURTHER INFORMATION SHEET PCT/ISA/210
2. ☒ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
see FURTHER INFORMATION SHEET PCT/ISA/210
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see FURTHER INFORMATION SHEET PCT/ISA/210

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-13 and 17-19 (all partially)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

- YES 1. Claims 1-13 and 17-19 (all partially): Use of 5-aza-2'-deoxycytidine for the manufacture of a medicament for restoring normal imprinting in eukaryotic cells.
- NO 2. Claims 1-13 and 17-19 (all partially): Use of trichostatin A for the manufacture of a medicament for restoring normal imprinting in eukaryotic cells.
- NO 3. Claims 1-13 and 17-19 (all partially): Use of lankacidin for the manufacture of a medicament for restoring normal imprinting in eukaryotic cells.
- NO 4. Claims 1-13 and 17-19 (all partially): Use of benzenamine for the manufacture of a medicament for restoring normal imprinting in eukaryotic cells.
- NO 5. Claims 1-13 and 17-19 (all partially): Use of cyclohexane acetic acid for the manufacture of a medicament for restoring normal imprinting in eukaryotic cells.
- NO 6. Claims 1-13 and 17-19 (all partially): Use of blue platinum uracil for the manufacture of a medicament for restoring normal imprinting in eukaryotic cells.
- NO 7. Claims 1-13 and 17-19 (all partially): Use of methyl 13-hydroxy-15-oxo-kaurenoate for the manufacture of a medicament for restoring normal imprinting in eukaryotic cells.
- NO 8. Claims 1-13 and 17-19 (all partially): Use of sulfonium for the manufacture of a medicament for restoring normal imprinting in eukaryotic cells.
- NO 9. Claims 1-13 and 17-19 (all partially): Use of euphomin D for the manufacture of a medicament for restoring normal imprinting in eukaryotic cells.
- NO 10. Claims 1-13 and 17-19 (all partially): Use of octadecylphosphoryl choline for the manufacture of a medicament for restoring normal imprinting in eukaryotic cells.
- NO 11. Claims 1-13 and 17-19 (all partially): Use of gnidimacrin for the manufacture of a medicament for restoring normal imprinting in eukaryotic cells.
- NO 12. Claims 1-13 and 17-19 (all partially): Use of aspiculamycin HCl for the manufacture of a medicament for restoring normal imprinting in eukaryotic cells.
- NO 13. Claims 14-15: Method for diagnosing the propensity of a mammal to develop cancer in a target organ or tissue.
- NO 14. Claim 16: Method to identify pharmacological agents involved in genomic imprinting.



**FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210**

Although claims 1-13 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

There is no method in claim 17: Claim 18 and 19 were searched for pharmaceutical composition instead of method.

The definition of chemical compounds by means of pharmacological parameters makes a complete search virtually impossible.

The compound 5-aza-2'-deoxycytidine has been considered to represent 5-aza-2'-deoxycytidine.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PLT/US 97/23991

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9215602 A	17-09-1992	CA 2104964 A EP 0631584 A US 5470970 A US 5688641 A US 5801001 A	29-08-1992 04-01-1995 28-11-1995 18-11-1997 01-09-1998
EP 0627436 A	07-12-1994	US 5616609 A AU 2409992 A CA 2114728 A WO 9303039 A	01-04-1997 02-03-1993 18-02-1993 18-02-1993
US 4497803 A	05-02-1985	JP 1671578 C JP 3036827 B JP 58177949 A CA 1216581 A DK 158883 A EP 0091782 A ZA 8302471 A	12-06-1992 03-06-1991 18-10-1983 13-01-1987 13-10-1983 19-10-1983 28-12-1983
US 5356903 A	18-10-1994	NONE	
US 4690918 A	01-09-1987	JP 1778962 C JP 4066453 B JP 61176523 A DE 3682091 A EP 0196415 A	13-08-1993 23-10-1992 08-08-1986 28-11-1991 08-10-1986

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(54) Title: <b>COMPOSITIONS AND METHODS FOR RESTORING A NORMAL PATTERN OF IMPRINTING TO CELLS</b>			
(57) Abstract <p>Compositions and methods for the diagnosis and treatment of diseases that are associated with imprinting are provided. Such compositions and methods are useful in restoring a normal pattern of imprinting to cells. The compositions comprise pharmacological agents that are involved in imprinting and can be used in preventive and therapeutic methods to restore a normal pattern of imprinting. Such agents are capable of restoring a normal pattern of imprinting to cells. Further methods of the invention include means for identifying pharmacological compounds that exert their effect by restoring normal imprinting to abnormally imprinted chromosomes and genes. Additionally, the invention could also be used to identify new compounds that exert their effect by restoration of normal imprinting to diseased tissues that exhibit underlying abnormal imprinting.</p>			

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## COMPOSITIONS AND METHODS FOR RESTORING A NORMAL PATTERN OF IMPRINTING TO CELLS

### FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with Government support under grants from the National Institutes of Health. The Government has certain rights to this invention.

### FIELD OF THE INVENTION

5 The present invention relates to compositions and methods of diagnosing, preventing and treating human diseases which are associated with parental origin-specific chromosome or gene alterations, particularly those diseases associated with abnormal patterns of imprinting. The present invention also relates to methods of discovering  
10 pharmacological agents which influence imprinting patterns of cells, particularly those which can restore a normal pattern of imprinting to abnormally imprinted cells.

### BACKGROUND OF THE INVENTION

Genomic imprinting is an epigenetic modification of a specific parental chromosome  
15 in the gamete or zygote that leads to monoallelic or differential expression of the two alleles of a gene in somatic cells of the offspring. Imprinting affects various essential cellular and developmental processes, including intercellular signaling, RNA processing, cell cycle control, and promotion or inhibition of cellular division and growth.

The first deduction of imprinting at the single gene level involved a transgenic C-  
20 myc gene which showed dependance of its expression on paternal inheritance. The silent maternally inherited copy was methylated (Swain *et al.* (1987) *Cell* 50:719-727).

-2-

Disruption of the insulin-like growth factor II (IGF2) gene in knockout experiments showed that IGF2 is imprinted and expressed normally only from the paternal allele, and that IGF2 is biparentally expressed in two neural tissues, the choroid plexus and the leptomeninges (DeChiara *et al.* (1991) *Cell* 64:849-859). These milestone studies showed that genomic imprinting affects normal endogenous genes and that imprinting shows tissue-specific regulation.

Direct approaches developed to identify novel imprinted genes include: positional cloning which identifies imprinted genes near other known imprinted genes (Barlow *et al.* (1991) *Nature* 349:84-87); comparing gene expression in parthenogenetic embryos to that of normal embryos (Kuroiwa *et al.* (1996) *Nat. Genet* 12:186-190); and restriction landmark genome scanning (Nagai *et al.* (1995) *Biochem. Biophys. Res. Commun.* 213:258-265). The last approach comprises analysis of clonality in tumors by assessing DNA methylation near a heterozygous polymorphic site (Vogelstein *et al.* (1985) *Science* 227:642-645).

Abnormalities of a single gene can affect imprinting of a proximate genomic region and disrupt multiple disease-causing genes, the phenotype depending upon the parental origin of the mutated gene. Two examples of imprinted human disease loci in close proximity are on chromosome 15. Disrupted imprinting of these loci is one of the causes of Prader-Willi syndrome (PWS) and Angelman syndrome (AS) which involve mental retardation. PWS also causes obesity, and AS involves gross motor disturbances. Each disorder can be caused by parental-origin specific uniparental disomy (Nicholls *et al.* (1989) *Nature* 342:281-285; Knoll *et al.* (1990) *Am. J. Hum. Genet.* 47:149-155). or chromosomal deletions (Knoll *et al.* (1989) *Am. J. Hum. Genet.* 47:149-155; Mattei *et al.* (1984) *Hum. Genet.* 66:313-334). The AS gene has not been isolated to date. PWS appears to be due to mutations or deletions in the small nuclear ribonucleoprotein polypeptide N (SNRPN) gene (reviewed in Nicholls *et al.* (1993) *Curr. Opin. Genet. Dev.* 3:445-446). A mutation affecting splicing of an untranslated upstream exon of SNRPN can also lead to AS, as well as abnormal imprinting of other loci (Dittrich *et al.* (1996) *Nat. Genet.* 14:163-170).

It is likely that many more additional imprinted human disease loci will be identified, because UPD for specific chromosomes is often associated with multiple

congenital anomalies (Ledbetter, D.H. and Engel, E. (1995) *Hum. Mol. Genet.* 4:1757-1764). Chromosomes that likely show this phenomenon include 2, 6, 7, 11, 14, 15, 16, 20, and X (Ledbetter, D.H. and Engel, E. (1995) *Hum. Mol. Genet.* 4:1757-1764).

5 Implication of genomic imprinting in cancer came from investigations of the two embryonic tumors, hydatidiform mole and complete ovarian teratoma, showing that not only 46 chromosomes are required to create a normal embryo, but also a balance of maternal and paternal chromosomes. A relative imbalance leads to neoplastic growth, and the type of neoplasm depends upon whether there is a maternal or paternal genetic excess.

10 Another tumor apparently associated with imprinting is familial paraganglioma or glomus tumor. In all cases, the transmitting parent is the father (Van der Mey *et al.* (1989) *Lancet* 2:1291-1294). The gene has recently been localized to 11q22.3-q23 (Heutink *et al.* (1994) *Eur. J. Hum. Genet* 2:148-158).

Loss of heterozygosity (LOH) in the childhood Wilms tumor occurs on chromosome 11 and the specifically involved region is 11p15 (Reeve *et al.* (1989) *Mol. Cell. Biol.* 9:1799-1803). Schroeder *et al.* (1987) *Am. J. Hum. Genet.* 40: 413-420, noted that in five of five cases of LOH, it was the maternal allele that was lost. This observation has been extended to other tumors of embryonal origin such as hepatoblastoma and rhabdomyosarcoma (Scrabble *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:7480-7484).

20 The N-myc gene on chromosome 2 shows preferential amplification of the paternal allele in neuroblastoma (Cheng *et al.* (1993) *Nature Genet.* 4:187-190). Advanced neuroblastoma tumors, showing N-myc amplification, also show preferential LOH of maternal chromosome 1, whereas earlier stage tumors without N-myc amplification do not (Caron *et al.* (1995) *Hum. Mol. Genet* 4:535-539). Thus, genetic disturbances involving imprinted genes in a given type of cancer may involve multiple chromosomes concurrently.

25 Naumova *et al.* (1994) *Am. J. Hum. Genet.* 54:274-281, found transmission ratio distortion, concordance of 13q loss and isochromosome 6 of the same parental origin in retinoblastoma, again consistent with a mechanism of generalized disturbance of imprinting in embryogenesis leading to increased cancer risk.

Beckwith-Wiedemann syndrome (BWS) is a disorder of prenatal overgrowth and cancer, transmitted as an autosomal dominant trait, or arising sporadically. A clue to a role for genomic imprinting is increased disease penetrance when BWS is inherited from the

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mother (Viljoen, D. and Ramesar, R. (1992) *J. Med. Genet* 29:221-225). The tumors of children show preferential loss of maternal 11p15 (Schroeder *et al.* (1987) *Am. J. Hum. Genet* 40:413-420; Scrabble *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:7480-7484), suggesting that an imprinted locus could cause BWS and also be involved in sporadically occurring tumors. Genetic linkage analysis showed that BWS localizes to 11p15, consistent with this idea of generalized disruption, and not to 11p13, to which the WT1 gene had been localized (Ping *et al.* (1989) *Am. J. Hum. Genet.* 44:720-723; Koufos *et al.* (1989) *Am. J. Hum. Genet.* 44:711-719).

More direct evidence for genomic imprinting of 11p15 in BWS came from studies showing that some patients with BWS have paternal UPD, involving a region extending from the  $\beta$ -globin locus to the RAS gene (Henry *et al.* (1991) *Nature* 35:609-610). While the large 10 Mb region does not provide precise localization of an imprinted gene, it provides a foundation for later studies of imprinted loci on this chromosome.

Examination of RNA from Wilms tumor (WT) led to a discovery that not one but both IGF2 alleles were expressed in 70% of Wilms tumors (Rainier *et al.* (1993) *Nature* 362:747-749; Ohlsson *et al.* (1993) *Nature Genet* 4:94-97). In addition, in 30% of cases, both alleles of H19 were expressed (Rainier *et al.* (1993) *Nature* 362:747-749). In contrast, examination of RNA from normal tissue show normal imprinting with the expression of one allele of IGF2 and H19. This was the first evidence of imprinting in humans. The term for this novel genetic alteration is loss of imprinting (LOI) (Rainier *et al.* (1993) *Nature* 362:747-749) which simply means loss of preferential parental origin-specific gene expression can involve either abnormal expression of the normally silent allele, leading to biallelic expression, or silencing of the normally expressed allele, leading to epigenetic silencing of the locus (Rainier *et al.* (1993) *Nature* 362:747-749; Feinberg (1993) *Nature Genet.* 4:110-113; Feinberg *et al.* (1995) *J. Natl. Cancer Inst. Monographs* 17:21-26). Thus, abnormal imprinting in cancer can lead to activation of normally silent alleles of growth-promoting genes (Rainier *et al.* (1993) *Nature* 362:747-749); (Feinberg (1993) *Nature Genet* 4:110-113); (Feinberg *et al.* (1995) *J. Natl. Cancer Inst. Monographs* 17:21-26).

Subsequently, LOI has been implicated in various tumor types. At first, LOI was found in other childhood tumors, such as hepatoblastoma (Rainier *et al.* (1995) *Cancer*



Res. 55:1836-1838); (Li *et al.* (1995) *Oncogene* 11:221-229). rhabdomyosarcoma (Zhan *et al.* (1994) *J. Clin. Invest.* 94:445-448), and Ewing's sarcoma (Zhan *et al.* (1995a) *Oncogene* 11:2503-2507). LOI of IGF2 and H19 have also now been found in many adult tumors, including uterine (Vu *et al.* (1995) *J. Clin. Endocrinol. Metab.* 80:1670-1676,  
5 cervical (Doucrazy *et al.* (1996) *Oncogene* 12:423-430), esophageal (Hibi *et al.* (1996) *Cancer Res.* 56:480-482), prostate (Jarrard *et al.* (1995) *Clin. Cancer Res.* 1:1471-1478), lung cancer (Kondo *et al.* (1995) *Oncogene* 10:1193-1198), choriocarcinoma (Hashimoto *et al.* (1995) *Nat. Genet.* 9:109-110), germ cell tumors (Van Gurp *et al.* (1994) *J. Natl. Cancer Inst.* 86:1070-1075) BWS (Steenman *et al.* (1994) *Nature Genet.* 7:433-439);  
10 Weksberg *et al.* (1993) *Nature Genet.* 5:143-150) and Wilms tumor (Ogawa *et al.* (1993) *Nature Genet.* 5:408-412). Thus, LOI is one of the most common genetic alterations in human cancer.

Another imprinted gene in 11p15 is p57<sup>KIP2</sup>, a cyclin-dependent kinase (CDK) inhibitor similar in its CDK inhibitory domain to p21<sup>WAF1/Cip1</sup>, a target of p53 (Matsuoka *et al.* (1995) *Genes Dev.* 9:640-662); Lee *et al.* (1995) *Genes Dev.* 9:639-649). The gene was  
15 localized within 40 kb of a group of BWS balanced germline chromosomal rearrangement breakpoints, in contrast to IGF2 and H19, which are located telomeric to those breakpoints (Hoovers *et al.* (1995) *Proc. Natl. Acad. Sci. USA* 92:12456-12460). Human p57<sup>KIP2</sup> was found to be imprinted with preferential expression from the maternal allele (Matsuoka *et al.* (1996) *Proc. Natl. Acad. Sci. USA* 93:3026-3030). p57<sup>KIP2</sup> also shows abnormal  
20 imprinting and epigenetic silencing in some tumors and BWS patients (Thompson *et al.* (1996) *Cancer Research* 56:5723-5727).

Cytosine DNA methylation is catalyzed by the enzyme cytosine (DNA-5)-methyltransferase (hereinafter, DNA methyltransferase). DNA methylation occurs almost  
25 exclusively at CpG dinucleotides (reviewed in Cedar *et al.* (1990) *Biochem. Biophys. Acta* 1049:1-8). The pattern of DNA methylation is heritable by somatic cells and maintained, after DNA replication, by DNA methyltransferase which has a 100-fold greater affinity for hemimethylated DNA (i.e., parent strand methylated, daughter strand unmethylated) than for unmethylated DNA (Cedar *et al.* (1990) *Biochem. Biophys. Acta* 1049:1-8). However,  
30 developing cells of the gamete and embryo undergo dramatic shifts in DNA methylation by both loss of methylation and de novo methylation (Cedar *et al.* (1990) *Biochem. Biophys.*

*Acta* 1049:1-8). The mechanism establishing the original pattern of DNA methylation is unknown.

Two classes of cytosine DNA methylation exist in the genome. The first, generally associated with gene silencing, occurs on genes that show tissue-specific expression. Since this type occurs both before and after the changes in gene expression, it is not necessarily the cause of altered gene expression during development; but rather may help to "lock in" a given pattern of gene expression (Cedar *et al.* (1990) *Biochem. Biophys. Acta* 1049:1-8); Riggs, A.D. (1989) *Cell Biophys.* 15:1-13). The second class involves CpG islands which are almost always unmethylated in normal cells, and are usually within the promoter or first exon of housekeeping genes (reviewed in Bird, A.P. (1986) *Nature* 321:209-213). However, an important exception is CpG islands on the inactive X-chromosome, which are methylated. Thus, CpG island methylation, unlike non-CpG island methylation, is thought to be involved in epigenetic silencing and/or marking of the inactive X chromosome (reviewed in Riggs, A.D. and Pfeifer, G.P. (1992) *Trends Genet.* 8:169-174).

Several lines of evidence provide a role for DNA methylation in the control of genomic imprinting. First, some imprinted genes in mice, such as H19, show parental origin-specific, tissue-independent methylation of CpG islands (Ferguson-Smith *et al.* (1993) *Nature* 362:751-755); Bartolomei *et al.* (1993) *Genes Develop.* 7:1663-1673. This methylation represents imprinting on the paternal chromosome and is not secondary to changes in gene expression. Second, knockout mice deficient in DNA methyltransferase, and exhibiting widespread genomic hypomethylation, do not show allele-specific methylation of the H19 CpG island and exhibit biallelic expression of H19 and loss of expression of IGF2 (Li *et al.* (1993) *Nature* 366:362-365). Similar parental origin-specific methylation has also been observed for a CpG island in the first intron of the maternally inherited, expressed allele of the IGF2 receptor gene (IGF2R) (Stoger *et al.* (1993) *Cell* 73:61-71). Methyltransferase-deficient knockout mice show loss of methylation of IGF2R and epigenetic silencing of the gene (Li *et al.* (1993) *Nature* 366:362-365).

Widespread alterations in DNA methylation in human tumors were discovered 15 years ago (Feinberg, A.P. (1983) *Nature Genet.* 4:110-113) and remain the most commonly found alteration in human cancers. These alterations are ubiquitous to both benign and malignant neoplasms (Goelz *et al.* (1985) *Science* 228:187-190). The precise

role of these changes remain unclear; but, both decreased and increased methylation have been found at specific sites in tumors, with an overall decrease in quantitative DNA methylation (Feinberg *et al.* (1988) *Cancer Res.* 48:1159-1161; Feinberg, A.P. (1988) *Prog. Clin. Biol. Res.* 279:309-317; Jones *et al.* (1990) *Adv. Cancer Res.* 54:1-23).

5 In humans, as in mice, the paternal allele of a CpG island in the H19 gene and its promoter is normally methylated, and the maternal allele is unmethylated (Steenman *et al.*, (1994) *Nature Genet* 7:433-439; Ferguson-Smith *et al.* (1993) *Nature* 362:751-755; Bartolomei *et al.* (1993) *Genes Develop.* 7:1663-1673). Because tumors with LOI of IGF2 showed reduced expression of H19, the methylation pattern of H19 has been examined in  
10 tumors with LOI. In all cases showing LOI of IGF2, the H19 promoter exhibits 90-100% methylation at the sites normally unmethylated on the maternally inherited allele (Steenman *et al.* (1994) *Nature* 7:433-439; Moulton *et al.* (1994) *Nature Genet.* 7:440-447). Thus, the maternal allele has acquired a paternal pattern of methylation, consistent with observed expression of IGF2 on the same maternally derived chromosome in these tumors. In  
15 contrast, tumors without LOI of IGF2 show no change in the methylation of H19, indicating that these changes are related to abnormal imprinting and not malignancy per se (Steenman *et al.* (1994) *Nature Genet* 7:433-439; Moulton *et al.* (1994) *Nature Genet.* 7:440-447). The same alterations in methylation of the maternal allele of H19 are found in BWS patients with LOI of IGF2 (Steenman *et al.* (1994) *Nature Genet.* 7:433-439; Reik *et al.* (1994) *Hum. Mol. Genet.* 3:1297-1301; Reik *et al.* (1995) *Hum. Mol. Genet.* 4:2379-  
20 2385).

A second potential mechanism of LOI may involve disruption of an imprinting control center on chromosome 11, similar to that recently described for the BWS/AS region of chromosome 15 (Dittrich *et al.* (1996) *Nat. Genet.* 14:163-170). A cluster of five  
25 BWS balanced germline chromosomal rearrangement breakpoints lies between p57<sup>KIP2</sup> on the centromeric side, and IGF2 and H19 on the telomeric side (Hoovers *et al.* (1995) *Proc. Natl. Acad. Sci. USA* 92:12456-12460). Thus, disruption of a gene spanning this region could cause abnormal imprinting, as well as BWS and/or cancer, at least when inherited through the germline.

30 Another potential mechanism for LOI involves loss of trans-acting factors which may establish and maintain a normal pattern of genomic imprinting once such a pattern is

established in the germline. Trans-acting modifiers of imprinting are likely to exist, since imprinting of transgenes is host strain-dependent (Sapienza *et al.* (1990) *Develop.* 107:165-168; Allen *et al.* (1990) *Cell* 61:853-361). Such genes might thus act as tumor suppressor genes in humans and other species.

- 5 Yet another potential mechanism of imprinting that might be disrupted in cancer involves histone deacetylation, which is linked to X-inactivation in mammals (reviewed in Wolffe, A.P. (1994) *Develop. Genet.* 15:463-470) and to telomere silencing in yeast (Thompson *et al.* (1994) *Nature* 369:245-247). Genes for both histone acetylase and histone deacetylase have recently been isolated (Brownell *et al.* (1996) *Cell* 84:843-851; 10 Taunton *et al.* (1996) *Science* 272:408-411). In addition, telomere silencing in yeast also involves the action of specific genes, e.g., SIR1-SIR4, some of which have homologues in mammals (Brachmann *et al.* (1995) *Genes Develop.* 9:2888-2902). Similarly, some examples of gene silencing in mammals may resemble position-effect variation in *Drosophila*, a form of position-dependent epigenetic silencing (Walters *et al.* (1996) *Genes 15 Develop.* 10:185-195). Finally, imprinted loci on maternal and paternal chromosomes may interact during DNA replication. Chromosomal regions harboring imprinted genes show replication and timing asynchrony (Kitsberg *et al.* (1993) *Nature* 364:459-463). Furthermore, the two parental homologues of some imprinted genes show nonrandom proximity in late S-phase (LaSalle, J.M. and Lalande, M. (1996) *Science* 272:725-728), 20 suggesting some form of chromosomal cross-talk, as has been observed for epigenetic silencing in *Drosophila* (Tatof, K.D. and Henikoff, S. (1991) *Cell* 65:201-203).

- The human IGF2 and H19 genes are normally imprinted, *i.e.*, show preferential expression of a specific parental allele (Rainier *et al.* (1993) *Nature* 362:747-749; Zhang, Y. and Tycko, B. (1992) *Nat. Genet.* 1:40-44; Giannoukakis *et al.* (1993) *Nat. Genet.* 25 4:98-101; Ohisson *et al.* (1993) *Nature Genet.* 4:94-97; Ogawa *et al.* (1993b) *Nature* 362:749-751). Furthermore, as discussed above, some tumors undergo loss of imprinting (LOI) in cancer, with one or more of the following: biallelic expression of IGF2 (Rainier *et al.* (1993) *Nature* 362:747-749; Ogawa *et al.* (1993b) *Nature* 362:749-751), epigenetic silencing of H19 (Steenman *et al.* (1994) *Nature Genet* 7:433-439; Moulton *et al.* (1994) 30 *Nature Genet.* 7:440-447); and/or abnormal expression of the paternal H19 allele (Rainier *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 85:6384-6388), and this observation has been

extended to a wide variety of childhood and adult malignancies (Rainier *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 85:6384-6388; Suzuki *et al.* (1994) *Nat. Genet.* 6:332-333). Normal imprinting can be maintained in part by allele-specific, tissue-independent methylation of H19, since LOI is associated with abnormal methylation of the normally unmethylated maternal H19 allele (Steenman *et al.* (1994) *Nature Genet.* 7:433-439; Moulton *et al.* (1994) *Nature Genet.* 7:440-447).

Alterations of genomic imprinting have been recently identified in human cancer, appearing commonly in both childhood and adult malignancies. While the art teaches abnormal imprinting and its association with disease states, there is no methods in the art for restoring normal imprinting to such cells. Thus, methods are needed in the art to restore a normal pattern of imprinting to cells.

#### SUMMARY OF THE INVENTION

Compositions and methods for the diagnosis and treatment of diseases that are associated with imprinting are provided. Such compositions and methods are useful in restoring a normal pattern of imprinting to cells. The compositions comprise pharmacological agents that are involved in imprinting. Such agents are capable of restoring a normal pattern of imprinting to cells. In this manner, the compositions and methods find use in the treatment of patients with diseases associated with abnormal imprinting. Such methods could also be used as diagnostic markers for identifying diseases, and disease subtypes, including certain cancers or malignant cell growth, in patients that would be responsive to treatment with agents to restore a normal pattern of imprinting.

Methods of the invention include means for identifying pharmacological compounds that exert their effect by restoring normal imprinting to abnormally imprinted chromosomes and genes. In this embodiment, methods are provided for testing drugs against reference cell lines (i.e., cell lines with either normal or abnormal imprinting) to determine which drugs, and lead compounds for developing other drugs, are useful for the diagnosis and treatment of imprinting-related diseases. Methods are provided for testing drugs against reference cell lines, such as those in the National Cancer Institute (NCI) database to determine which drugs are useful for the diagnosis and treatment of imprinting-related

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diseases. Additionally, such compounds, compositions comprising such compounds, and methods for their use are encompassed.

The invention is further drawn to methods for determining the propensity of an individual to develop cancer or a malignant cell growth. Such diagnosis involves  
5 determining the pattern of imprinting for cells of interest. The determination can be followed by preventive therapies where abnormal imprinting is found. In these instances the methods of the invention can be used to treat malignant cells as well as to restore a normal pattern of imprinting to normal, or pre-disease, cells that exhibit an abnormal pattern of imprinting.

10 In the same manner, using the methods of the invention, a regimen specialized for the treatment of a particular malignant growth in a particular individual can be developed. Such individualized regimens are useful for the treatment of cancers or malignancies with minimal damage to normal cells.

Pharmaceutical compositions are provided which are useful in the diagnosis and  
15 treatment of human diseases associated with abnormal imprinting, particularly those that are related to parental origin-specific chromosome or gene alterations, such as many types of cancer or organ specific malignant cell growth. It is important to note that the invention encompasses pharmaceutical compositions which are useful in the diagnosis and treatment of imprinting-related cancers or malignant cell growth for which the underlying imprinting  
20 mechanism has not yet been elucidated. In other words, the invention encompasses the successful diagnosis and treatment of any cancer, by the inhibition of malignant growth, which responds to the disclosed treatment methodology.

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#### **BRIEF DESCRIPTION OF THE DRAWINGS**

The invention is better understood by reading the following detailed description with reference to the accompanying figures, in which like reference numerals refer to like elements throughout, and in which:

30 **Figure 1.** Figure 1 depicts the effects of 5-azaCdR on imprinting of tumor cells with LOI. A) Switch to preferential allelic expression of IGF2. RT-PCR was performed

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on total RNA extracted from JEG-3 cells after a single 24 hr treatment with 0, 0.3, 0.6, or 1.0  $\mu$ M 5-azaCdR (indicated). Alternating lanes represent simultaneous experiments with and without reverse transcriptase. PCR products were digested with Apa I. The a and b alleles are 236 and 173 bp, respectively. B) Induction of H19 expression. Expression of U19 in JEG-3 cells after treatment with 5-azaCdR was measured by Northern blot analysis. Blots were rehybridized with GAPDH as a control for loading. C) Switch to monoallelic expression of H19. RT-PCR was performed as in (A) above. PCR products were digested with Alu I to detect allele-specific expression. The a and b alleles are 227 and 100 bp, respectively. Please note that the RT-PCR assay is not quantitative, and the total amounts of RNA in the three sets were not normalized.

**Figure 2.** Figure 2 depicts the effects of 5-azaCdR on H19 promoter methylation. (A) Decreased methylation of H19 after 5-azaCdR treatment. DNA was extracted from cells treated with 0, 0.3, or 1.0  $\mu$ M 5-azaCdR, and digested with Pst I and Sma I to assay DNA methylation as described. The 1.8 kb fragment represents methylated DNA and the 1.0 kb fragment unmethylated DNA. (B) Allele specificity of altered methylation. BamH I and Hpa II digested DNA was PCR-amplified and digested with Alu I to detect allele-specific methylation. The A and B alleles (shown) are 227 and 100 bp, respectively.

**Figure 3.** Figure 3 depicts a unified model of LOI occurring in some cancers, such as Wilms tumor, which explains both epigenetic silencing of tumor suppressor genes, as well as activation of normally silent alleles of growth-promoting genes. According to this model, LOI involves a switch in the epigenotype of a chromosomal region) for example, in the case of Wilms tumor from maternal to paternal. Thus, IGF2 shows biallelic expression and H19 undergoes epigenetic silencing. However, this model is not meant to be universal. For example, not all tumors show a switch in expression of all three genes, IGF2, H19 and p57<sup>KIP2</sup>. Hepatoblastoma shows LOI of IGF2, but tumors with biallelic expression of IGF2 do not necessarily undergo epigenetic silencing of H19. In fact, it is recognized that now that the present invention has demonstrated that a normal pattern of imprinting can be restored in cells exhibiting LOI, a number of pathways or mechanisms will become available. Thus, the present invention is not limited to any particular mechanism of restoration of a normal pattern of imprinting.

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More specifically, Figure 3 shows an imprint organizing center (red rectangle), exerts a long-range cis-acting influence on IGF2 and other imprinted genes (blue ellipses), via alterations in chromatin structure (represented as DNA loops). This imprint organizing center establishes the imprinting mark as maternal or paternal. This effect is propagated outward during development, similar to the organizing center on the X-chromosome. Imprinting is maintained in part by allele-specific methylation of CpG islands, as well as interactions with trans-acting proteins (green circles). According to this model, loss of imprinting could arise by any of several mechanisms (numbered in the figure): (1) Deletion or mutation in the imprint organizing center itself, which would lead to a failure of parental origin-specific switching in the germline; (2) separation of the imprint organizing center from the imprinted target genes, as seen in BWS germline chromosomal rearrangement cases; (3) abnormal methylation of CpG islands; (4) local mutation of regulatory sequences controlling the target imprinted genes themselves; or (5) loss of or mutations in genes for trans-acting factors that maintain normal imprinting. Figure modified from Feinberg *et al.* (1994) Cambridge University Press, 19:273-292, which is herein incorporated in its entirety by reference.

### DETAILED DESCRIPTION OF THE INVENTION

Compositions and methods for restoring a normal pattern of imprinting to cells with abnormal imprinting patterns are provided. The compositions comprise pharmacological agents that are involved in and capable of restoring a normal pattern of imprinting to cells. The compositions and methods of the invention can be used to restore normal imprinting in any cell type. Of particular interest are eukaryotic cells, particularly plant and mammalian cells, more particularly human cells.

Imprinting has been observed in eukaryotic cells of plants and mammals (Yoder and Bestor (1996) *Biol. Chem.* 377(10): 605-610). In humans and other mammals, normal imprinting underlies several fundamental cellular and developmental processes; thus, abnormal imprinting patterns are implicated in a wide variety of catastrophic human diseases. Restoration of normal imprinting patterns, as provided by the present invention, is useful for diagnosis, prevention and therapy of diseases associated with abnormal imprinting. Imprinting is defined as an epigenetic modification of a specific parental allele



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of a gene, or the chromosome on which it resides, in the gamete or zygote, leading to differential expression of the two alleles in somatic cells of the offspring.

The methods of the invention can be used to restore a normal pattern of imprinting to cells. "Normal pattern of imprinting" means preferential expression of a single parental allele of an imprinted gene and/or preferential methylation of a single parental allele of an imprinted gene. "Loss of imprinting or LOI" means loss of a normal pattern of imprinting, i.e., the loss of preferential expression of a single parental allele of an imprinted gene and/or the loss of methylation of a single parental allele of an imprinted gene. LOI is exhibited by a variety of abnormal expression patterns. Such patterns include but are not limited to: equal expression of both alleles; significant (>5%) expression of the normally silent allele when the normal case is complete silencing of one allele; epigenetic silencing of the normally expressed copy of an imprinted gene; the absence of methylation of both alleles and/or the methylation of both alleles where the normal case is methylation of a single allele.

The compositions and methods of the invention work to restore such normal patterns of imprinting. Such methods and compositions are capable of modulating both the extent and the pattern of gene expression.

Thus, for purposes of the invention, "restoration of normal imprinting" means restoring preferential expression of a single parental allele of an imprinted gene. It is recognized that various mechanisms are available for restoring normal imprinting and all such mechanisms are encompassed herein. Such mechanisms include: the relative silencing of one copy of an allele pair where both alleles were expressed at significant-comparative levels prior to treatment; the restoration of preferential expression of the same parental allele that is normally expressed; the preferential expression of a specific allele in a pattern opposite to that seen in normal tissues (allele switching); the re-expression of one copy of an imprinted gene where both copies had been silenced; the restoration of a normal pattern of methylation to allele pairs, i.e., where one allele is methylated and the other allele is not, where abnormal imprinting was exhibited by the absence of methylation on both alleles or alternatively by the methylation of both alleles; and other mechanisms. The importance of each mechanism is that the preferential expression of a single parental allele is restored.

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The compositions and methods of the invention are useful in the treatment of diseases associated with abnormal imprinting. In some instances, the genes exhibiting abnormal imprinting have been elucidated. In cancer, in particular, a number of genes exhibit loss of imprinting with epigenetic silencing of the normally expressed allele. Such genes include, but are not limited to, H19, IGF2, p57<sup>KIP2</sup> (Matsuoda *et al* (1996) *Proc. Natl. Acad. Sci. USA* 93:3026-3030; and Thompson *et al.* (1996) *Cancer Res.* 56:5723-5727); TSSC3; KvLQT1 (Lee *et al.* (1997) *Nature Genetics* 15:181-185 Rainier *et al.* (1993) *Nature* 362:747-749). However, the methods of the invention are not dependent on knowing the gene involved in the disease. The compositions and methods of the invention work to restore a normal pattern of imprinting even where the responsible gene or genes are unknown.

The methods of the invention find use in restoring a normal pattern of imprinting to cells involved in a range of diseases affecting many tissues. To date, such diseases include Prader Willi and Angelman syndromes caused by parental-specific disomies (Nicholls *et al.* (1989) *Nature* 342: 281-285; Knoll *et al.* (1989) *Am. J. Med. Genet* 32: 285-290; Knoll *et al.* (1990) *Am. J. Hum. Genet.* 47: 149-155); Mattei *et al.* (1984) *Hum. Genet* 666: 313-334; Nicholls *et al.* (1993) *Curr. Opin.. Genet. Dev.* 3: 445-446; Dittrich *et al.* (1996) *Nat. Genet.* 14: 163-170; Ledbetter *et al.* (1995) *Hum. Mol. Genet.* 4: 1757-1764); myotonic dystrophy (DM), Huntington's disease (HD), spinocerebellar ataxia type 1 (SCA 1), fragile X syndrome (FRAXA) and neurofibromatosis types 1 and 2 (Chatkupt *et al.* (1995) *J. Neurol. Sci* 130: 1-10); schizophrenia (Ohara *et al.* (1997) *Biol. Psychiatry* 42(9): 760-766) ; autoimmune disease (Politaev and Selifanova (1994) *Life Sci* 54:1377-1381; Kerzin Storrar *et al.* (1988) *Neurology* 38: 38-42).

Additionally, loss of normal imprinting patterns are specifically related to oncogenesis and progression of particular cancers (Rainier *et al.* (1993) *Nature* 362: 747-749; Feinberg *et al.* (1993) *Nature Genet.* 4: 110-113; Feinberg *et al.* (1995) *J. Natl. Cancer Inst. Monographs* 17: 21-26), including Wilm's tumor (Ogawa *et al.* (1993) *Nature* 362: 749-751); rhabdomyosarcoma (Zhan *et al.* (1994) *J. Clin. Invest.* 94: 445-448), Ewing's sarcoma (Zhan *et al.* (1995) *Oncogene* 11:2503-2507); germ cell tumors (Van Gurp *et al.* (1994) *J. Natl. Cancer Inst.* 86:1070-1075); choriocarcinoma (Hashimoto *et al.* (1995) *Nature Genet.* 9:109-110); lung cancer (Suzuki *et al.* (1994) *Nat. Genet.* 6: 332-

333); hepatoblastoma (Rainier et al. (1995) *Cancer Res.* 55: 1836-1838); hydatidiform mole (Kajii and Ohama (1977) *Nature* 268:633); parthanogenetic ovarian tetromas (Linder et al. (1975) *N. Eng. J. Med.* 292:63-66); paraganglioma or glomus tumor (Van Der May et al. (1989) *Lancet* 2:1291-1294); neuroblastoma (Cheng et al. (1993) *Nature Genet.* 5: 4:187-190); retinoblastoma (Naumova et al. (1994) *Am. J. Hum. Genet.* 54: 274-281); leukemia (Momparker et al. (1997) *Anti-Cancer Drugs* 8: 358-368; Petti et al. (1993) *Leukemia* 7(Supp. 1): 36-41); hepatocellular cancer and their normal tissue (Takeda et al. (1996) *Oncogene* 12:1589-1592); esophageal cancer (Hibi et al. (1996) *Cancer Research* 56:480-482); lung cancer (Suzuki et al. (1994) *Nature Genetics* 6:332-333 and Kondo et al. (1995) *Oncogene* 10:1193-1198); adrenocortical carcinoma, breast cancer, bladder cancer, ovarian cancer, colon cancer, testicular cancer, prostate cancer (Hu et al. (1997) *Genomics* 46:9-17, Jarrard et al. (1995) *Clin. Cancer Res.* 1:1471-1478); and Beckwith-Wiedemann syndrome (BWS) (Henry et al. (1991) *Nature* 35: 609-610). Also included are melanomas, leukemias, and cancers of ovary, kidney, and central nervous system origin.

15 It is recognized that additional cancers and/or diseases will be associated with abnormal imprinting. The methods of the invention will be applicable to these diseases where a normal pattern of imprinting can be restored in such cells.

LOI can involve activation of the normally silent copy of growth-promoting genes, silencing of the normally transcribed copy of tumor suppressor genes, or both. These changes can involve a switch of one chromosome to the epigenotype of the other chromosome. For example, the maternal chromosome can be switched to the epigenotype of the paternal chromosome.

Compositions of the invention comprise pharmacological agents that are capable of altering the imprinting patterns of a cell. Such pharmacological agents include agents involved in DNA methylation; such as inhibitors of DNA methyltransferases, topoisomerase II, DNA synthesis, microtubule formation, histone deacetylation, and the like. (Wachsman (1997) *Mutat Res* 375 (1): 1-8). Specific examples of such agents include 5-azaCdR, Trichostatin A, lankacidin, benzenamine, blue platinum uracil, cyclohexane acetic acid and methyl 13-hydroxy-15-oxo-kaurenoate, etc.

30 Methods to identify additional pharmacological agents involved in genomic imprinting are provided. Of particular interest is identification of those agents that are

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capable of restoring a normal pattern of imprinting to abnormally imprinted cells. Methods for identification of such agents include administering a pharmacological agent to cells, and determining the pattern of imprinting of the cells in comparison to that of a pretreatment period or a reference cell line. A number of human cancer cell lines are available for testing the effect of imprinting by agents of interest. See, for example, Weinstein *et al.* (1997) *Science* 275:343-349, herein incorporated by reference. The administering of the agent of interest can be alone or in conjunction with other agents. Cells can be selected based on their propensity to a known agent capable of restoring a normal pattern of imprinting to abnormally imprinted cells. See, for example, Experimental herein.

10 It is further recognized that combinatorial approaches to testing agents involved in imprinting can be utilized. Thus, methods for selecting chemical compounds that are capable of restoring a normal pattern of imprinting to cells are provided. Such methods comprise administration of compounds to a drug discovery program system as disclosed in Weinstein *et al.* (1997) *Science* 275:343-349. Compounds to be tested for ability to restore  
15 a normal pattern of imprinting are administered to such cells. Those compounds showing reversal of LOI are selected for further testing.

The compounds to be tested can be organic compounds, peptides, peptides containing chemical modifications or substituents, and the like. Methods for synthesizing such compounds are available in the art. Combinatorial chemistry approaches can be  
20 utilized. Such approaches increase the range of molecular diversity available for selection and greatly broaden the scope of random collections of molecules being surveyed for biological activity. See, generally, John N. Abelson (ed.) *Methods in Enzymology, Combinatorial Chemistry*, Vol. 267, Academic Press (1996), herein incorporated by reference. In the same manner, once lead compounds are identified they can be modified  
25 using such combinatorial chemistry methods to select for compounds or agents having greater effect or stability.

Peptides can be synthesized at random and subsequently tested for those which are involved in imprinting. Methods for the creation of such random peptide libraries are known. See, for example, US Patent Nos. 5,270,170; 5,498,530; and H. M. Sassenfeld  
30 (1990) *TIBTECH* 8:88-93; and the references cited therein; all of which are herein incorporated by reference.

- The same effect may also be achieved by gene delivery of a portion of a protein exhibiting activity. Such vectors for gene delivery are available in the art and include SV40 virus (See, e.g., Okayama *et al.* (1985) *Molec. Cell Biol.* 5:1136-1142); Bovine papilloma virus (See, e.g., DiMaio *et al.* (1982) *Proc. Natl. Acad. Sci. USA* 79:4030-4034); adenovirus (See, e.g., Morin *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:4626; Yifan *et al.* (1995) *Proc. Natl. Acad. Sci. USA* 92:1401-1405; Yang *et al.* (1996) *Gene Ther.* 3:137-144; Tripathy *et al.* (1996) *Nat. Med.* 2:545-550; Quantin *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:2581-2584; Rosenfeld *et al.* (1991) *Science* 252:431-434; Wagner (1992) *Proc. Natl. Acad. Sci. USA* 89:6099-6103; Curiel *et al.* (1992) *Human Gene Therapy* 3:147-154; Curiel (1991) *Proc. Natl. Acad. Sci. USA* 88:8850-8854; LeGal LaSalle *et al.* (1993) *Science* 259:590-599; Kass-Eisler *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:11498-11502); adeno-associated virus (See, e.g., Muzyczka *et al.* (1994) *J. Clin. Invest.* 94:1351; Xiao *et al.* (1996) *J. Virol.* 70:8098-8108); herpes simplex virus (See, e.g., Geller *et al.* (1988) *Science* 241:1667; Huard *et al.* (1995) *Gene Therapy* 2:385-392; U.S. Patent No. 5,501,979); retrovirus-based vectors (See, for example, Curran *et al.* (1982) *J. Virol.* 44:674-682; Gazit *et al.* (1986) *J. Virol.* 60:19-28; Miller, A.D. (1992) *Curr. Top. Microbiol. Immunol.* 158:1-24; Cavanaugh *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:7071-7075; Smith *et al.* (1990) *Molecular and Cellular Biology* 10:3268-3271); herein incorporated by reference.
- In the same manner, the peptides can be used in any mammalian expression vector. See, for example, Wu *et al.* (1991) *J. Biol. Chem.* 266:14338-14342; Wu and Wu (*J. Biol. Chem.* (1988)) 263:14621-14624; Wu *et al.* (1989) *J. Biol. Chem.* 264:16985-16987; Zenke *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87:3655-3659; Wagner *et al.* (1990) 87:3410-3414.
- Standard techniques for the construction of the vectors of the present invention are well-known to those of ordinary skill in the art and can be found in such references as Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd ed. (Cold Spring Harbor, New York, 1989). A variety of strategies are available for ligating fragments of DNA, the choice of which depends on the nature of the termini of the DNA fragments and which choices can be readily made by those of skill in the art.

Methods for the determination of the pattern of imprinting are known in the art. It is recognized that the methods may vary depending on the gene to be analyzed. Generally, in methods for assaying allele-specific gene expression, RNA is reverse transcribed with reverse transcriptase, and then PCR is performed with PCR primers that span a site within an exon where that site is polymorphic (i.e., normally variable in the population), and this analysis is performed on an individual that is heterozygous (i.e., informative) for the polymorphism. One then uses any of a number of detection schemes to determine whether one or both alleles is expressed. Methods for the assessment of gene expression, allele specific gene expression, and DNA methylation are encompassed (see Experimental section herein). Additionally, direct approaches to identifying novel imprinted genes include: positional cloning efforts aimed at identifying imprinted genes near other known imprinted genes (Barlow *et al.* (1991) *Nature* 349:84-87); techniques comparing gene expression in parthenogenetic embryos to that of normal embryos (Kuroiwa *et al.* (1996) *Nat. Genet.* 12:186-190); and restriction landmark genome scanning (Nagai *et al.* (1995) *Biochem. Biophys. Res. Commun.* 213:258-265). See also, Rainier *et al.* (1993) *Nature* 362:747-749; which teaches the assessment of allele-specific expression of IGF2 and H19 by reverse-transcribing RNA and amplifying cDNA by PCR using new primers that permit a single round rather than nested PCR; Matsuoka *et al.* (1996) *Proc. Natl. Acad. Sci USA* 93:3026-3030 which teaches the identification of a transcribed polymorphism in p57<sup>KIP2</sup>; Thompson *et al.* (1996) *Cancer Research* 56:5723-5727 which teaches determination of mRNA levels by RPA and RT-PCR analysis of allele-specific expression of p57<sup>KIP2</sup>; and Lee *et al.* (1997) *Nature Genetics* 15:181-185 which teaches RT-PCR SSCP analysis of two polymorphic sites. Such disclosures are herein incorporated by reference.

Once agents are identified they can be used in methods for treatment or prevention of cancer, that is malignant growth of a tissue or organ, and other diseases associated with abnormal imprinting as noted above. Such methods include administering effective amounts of a pharmacological agent capable of restoring a normal pattern of imprinting to a patient in need thereof. The need for treatment will be based on the observation that the patient exhibits both the underlying abnormal pattern of imprinting in the target cells, as well as the symptoms associated with the particular disease. The need for prevention will

be based on the observation of the underlying abnormal imprinting pattern in the target cells.

By "effective amount" is intended that amount to induce a concentration of the agent in the cells of the target organ or tissue needed to restore a normal pattern of imprinting to those cells. Such effective amount may vary from agent (or compound) to agent. It has been shown that such effective amounts can be administered to cancer cells in culture such that cytotoxicity of normal cells, a secondary effect, can be kept to a minimum. For example, such results were obtained with tumor cells cultured in 0.3-1.0  $\mu$ M 5-azaCdR for 24 hours (see Experimental Section herein). It should be emphasized that while some of the same or similar agents described herein can exhibit anti-cancer activity through general cytotoxic effects, the present invention describes methods and compositions in which the agents exert their primary effect distinctly and separately from general nonspecific cytotoxic effects on target cells with abnormal imprinting. Doses of a particular agent may be predicted based on doses that have been used with tumor cells in vitro. Where the compounds or agents have not been used before, predictions can be made based on its relationship to other drugs which have been used. Predicted ranges of a particular agent may be further tested on cells that correspond to target cells in vitro to determine the concentration needed to alter abnormal imprinting. Such effective concentrations can be used to extrapolate appropriate dosages and methods of administration in the patient.

While the invention is not bound by such, generally where the drugs have been used to exert cytotoxic effects, the amounts encompassed here for an effective amount will be below that taught in the art. For example, with 5-aza-2'-deoxycytidine the prior art teaching for treatment with leukemia is from 0.86 to 1.3 mM. The effective amount of the same agent for purposes of the present invention is from about 0.3 to about 1.0  $\mu$ M, a one thousand fold lower dosage than that taught in the art.

It is recognized that the effective amount of the agent will vary with the disease type and extent, pharmacological parameters of each specific agent, and the responsiveness of individual patients to treatment with particular agents.

The methods of the invention can be used to formulate an individual regimen scheme for malignant growths or cancers. The scheme is based on the determination of effective agents for a particular cancer type in a particular patient, in light of the above

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variables. For example, cancerous and normal tissue obtained from the patient can be used to determine the imprinting patterns of the cells. Cell lines or primary cultures established from the obtained tissue can be used to determine the type and effective concentrations of agents in the target cells that can restore a normal imprinting pattern to the cells as well as inhibit the growth of the malignant cells. This allows for providing effective regimens with minimum cytotoxicity to normal cells. It is recognized in the art that particular agents or drugs are effective for some cancers in particular patients but not in others. The methods herein allow the physician to design a regimen particularly suited to an individual.

The compositions of the invention may be provided as pharmaceutical formulations suitable for parenteral (e.g., subcutaneous, intradermal, intramuscular, intravenous and intraarticular) administration. Formulations of the present invention comprise the pharmacological agent in a physiologically or pharmaceutically acceptable carrier, such as an aqueous carrier. Such administrations provide for systemic or alternatively for perfusion to particular tissues or organs. The formulations may be conveniently prepared in unit dosage form and may be prepared by any of the methods well-known in the art. Such formulations are described in, for example, *Remington's Pharmaceutical Sciences* 19th ed., Osol, A. (ed.), Mack Easton PA (1980), herein incorporated by reference. The most suitable route of administration in any given case may depend upon the subject, the nature and severity of the condition being treated, and the particular active compound which is being used.

The present invention provides for the use of the pharmacological agent having the characteristics set forth above for the preparation of a medicament for the various disorders. In the manufacture of a medicament according to the invention, the agent or agents are typically admixed with, *inter alia*, an acceptable carrier. Any inert pharmaceutically-acceptable carrier may be used, such as sterile saline or water of acceptable purity, or phosphate-buffered saline, or any such carrier in which the compositions of the present invention have suitable solubility properties for use in the methods of the present invention. The carrier must, of course, be acceptable in the sense of being compatible with any other ingredients in the formulation and must not be deleterious to the patient. The carrier may be a solid or a liquid. One or more agents of the invention may be incorporated in the formulations of the invention, which may be prepared by any of the well known techniques of



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pharmacy consisting essentially of admixing the components, optionally including one or more accessory therapeutic ingredients.

Aqueous and non-aqueous sterile suspensions of the formulation may include suspending agents and thickening agents. The formulations may be presented in unit dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, saline or water-for-injection immediately prior to use.

Additional pharmaceutical methods may be employed to control the duration of action. Controlled release preparations may be achieved by the use of polymers to complex or absorb the compositions. The controlled delivery may be exercised by selecting appropriate macromolecules (for example, polyesters, polyamino acids, polyvinyl pyrrolidone, ethylene-vinylacetate, methylcellulose, carbosymethylcellulose, or protamine sulfate). The rate of drug release may also be controlled by altering the concentration of such macromolecules.

Another possible method for controlling the duration of action comprises incorporating the therapeutic agents into particles of a polymeric substance such as polyesters, polyamino acids, hydrogels, poly(lactic acid) or ethylene vinylacetate copolymers. Alternatively, it is possible to entrap the therapeutic agents in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, by the use of hydroxymethyl cellulose or gelatin-microcapsules or poly(methylmethacrylate) microcapsules, respectively, or in a colloid drug delivery system, for example, liposomes, albumin, microspheres, microemulsions, nanoparticles, nanocapsules, or in macroemulsions. Such teachings are disclosed in *Remington's Pharmaceutical Sciences* (1980).

The dosage of the agent administered will depend upon the particular method being carried out, and when it is being administered to a subject, will depend on the disease, the condition of the subject, the particular formulation, the route of administration, etc. In general, the amount of the compound will be calculated based on the concentration of the compound needed in the cells to effectively restore a normal pattern of imprinting as discussed above. This amount will vary from compound to compound.

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The compositions of the invention can be used alone or in combination with other drugs and therapeutic agents for the treatment of cancer and malignant cell growth as well as with other agents useful in the treatment of other diseases associated with abnormal imprinting. Other agents are known in the art.

5 In one model of cancer or malignant growth, it is postulated that the normal apoptotic pathway of the malignant cell has been disrupted. Thus, the cell does not die after a mutation but rather survives and propagates. Abnormal imprinting can inhibit apoptosis (Christofori *et al.* (1994) *Nature* 369:414-418 and Christofori *et al.* (1995) *Nature Genetics* 10:196-201). While not limited to this hypothesis herein, the agents and  
10 methods of the invention may aid in restoring the apoptotic pathway in these cells by restoring a normal pattern of imprinting. In this manner, the agents of the invention will act to enhance the effect of other drugs or agents in treating the tumor cells.

The methods of the invention are also useful for diagnosis and typing of diseases that are associated with an abnormal pattern of imprinting, and for determining whether  
15 such diseases will respond to therapy following the administration of agents that restore a normal pattern of imprinting. For example, the work herein demonstrates that patients with colorectal cancer exhibit LOI in the cancerous tissue, but the normal mucosal cells of these cancer patients also exhibit LOI. Thus, it appears that LOI precedes the development of cancer and can be used to predict the propensity of an individual to the development of  
20 cancer or malignant or tumor growth.

Thus, the methods of the invention can be used to determine the propensity of an individual to develop cancer. In those instances where an abnormal pattern has been detected, the pharmacological agents of the invention can be used to prevent malignant growth or the onset of the disease. Such administration will lead to a restoration of a  
25 normal pattern of imprinting and normal monoallelic expression. As more information becomes available regarding associations of specific imprinted loci with particular diseases, the methods of the invention will gain wider applicability in determining the propensity of an individual to such diseases as well as providing effective therapies for the prevention and treatment of such diseases.

30

## EXPERIMENTAL

**Example 1: Reversal of loss of imprinting in tumor cells.****Materials and Methods:**

Cell Culture and Treatment With 5-azaCdR. JEG-3 human choriocarcinoma and COLO-205 human colon adenocarcinoma cell lines were obtained from ATTC. Cells were  
5 cultured in RPMI 1640, 10% fetal calf serum, 5% CO<sub>2</sub>, with antibiotics. 10<sup>4</sup>-10<sup>5</sup> cells in 6 cm dishes were treated with 0.3-10  $\mu$ M 5-azaCdR for 24 hours. Cultures were washed in Hank's buffered saline solution and grown to confluence. Concentrations of 0.3-3.0  $\mu$ M caused minimal cytotoxicity (0-30%).

Analysis of Gene Expression. For quantitative analysis of gene expression, total  
10 RNA was isolated as described (Chomczynski, P. and Sacchi, N. (1987) *Anal. Biochem.* 162:156-159), and 10  $\mu$ g was electrophoresed in 1.2% agarose gels containing formaldehyde, and was transferred to Hybond-N+ membranes (Amersham) and hybridized as described with H19 and IGF2 probes (Steenrnan *et al.* (1994) *Nature Genet.* 7:433-439). Hybridization with GAPDH was used as a control for RNA loading. For analysis of allele-  
15 specific expression, 0.5  $\mu$ g RNA was treated with DNase I (Boehringer Mannheim), heated to 95°C for 7 minutes, and RT-PCR was performed as described for both IGF2 and H19 (Rainier *et al.* (1993) *Nature* 362:747-749), although nested PCR using primers HP1 and HP2 (Hashimoto *et al.* (1995) *Nat. Genet.* 9:109-110) was performed for H19 analysis. PCR products were digested with either Apa I (for IGF2 analysis) or Alu I (for H19  
20 analysis) as described (Tadokoro *et al.* (1991) *Nucleic Acids Res.* 19:6967), and electrophoresed on 4% Nusieve-agarose (3:1) gels. Each sample was analyzed in duplicate in the presence and absence of reverse transcriptase to exclude DNA contamination. Gels were blotted and hybridized with a <sup>32</sup>P-labeled internal specific oligonucleotide probe (Rainier *et al.* (1993) *Nature* 362:747-749), and quantified with a PhosphorImager  
25 (Molecular Dynamics).

Analysis of DNA Methylation. For quantitative analysis of DNA methylation, genomic DNA was prepared as described (Rainier *et al.* (1993) *Nature* 362:747-749), and Southern analysis of methylation of the H19 promoter CpG island was performed as described (Reik *et al.* (1994) *Hum. Mol. Genet.* 4:2379-2385). Filters were hybridized  
30 with the 1.8 kb Pst I fragment of H19, which detects imprint-specific methylation

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(Steenman *et al.* (1994) *Nature Genet.* 7:433-439). For analysis of allele-specific methylation, 1 µg DNA was digested with BamH I or with BamH I plus Hpa

After digestion, enzymes were inactivated by heating to 100° for 10 min. 50 ng digested DNA was added to the PCR reaction mix in Buffer F (Invitrogen), containing  
5 primers HP1 (5'-TACAACC-ACTGCACTACCTG-3') and H3 (5'-TGGAATGCTTGAAGGCTGCT-3') (Hashimoto *et al.* (1995) *Nat. Genet.* 9:109-110). One initial cycle (94°C, 4 min.) was followed by 28 cycles of 94°C, 1 min., 60°C, 1 min., 72° C, 1 min. PCR products (227 bp) were digested with Alu I, electrophoresed on 3% Nusieve/1% agarose gels, transferred to Hybond-N+ membranes (Arnersham), and  
10 hybridized with an internal oligonucleotide probe HP2 (5'-TGGCCATGAAGATGGAGTCG-3'), and then quantified with a PhosphorImager (Molecular Dynamics).

#### Results:

15 The purpose of the experiments described here was to determine whether any of the features of loss of imprinting (LOI) in tumor cells was reversible with 5-aza-2'-deoxycytidine (5-azaCdR), a specific inhibitor of DNA methylation. We chose as a model system two tumor cell lines, JEG-3 choriocarcinoma and COLO-205 colorectal carcinoma cells, by screening cell lines for those that fulfilled two criteria: they were heterozygous for  
20 transcribed polymorphisms in IGF2 and H19, and thus informative for imprinting studies; and they showed LOI. Cells were treated with 5-azaCdR for 24 hours (approximately 1 cell division) and at concentrations experimentally determined both in our laboratory (Rainier, S. and Feinberg, A.P. (1988) *Proc. Natl. Acad. Sci. USA* 85:6384-6388) and others (Taylor, S.M. and Jones, P.A. (1979) *Cell* 17:771-779) to maximize hypomethylation and  
25 minimize cytotoxicity.

We first examined the effect of 5-azaCdR on LOI of IGF2. Tumor cells with LOI show equal biallelic expression of IGF2, in contrast to cells with normal imprinting, which exhibit preferential expression of a specific parental allele (Rainier *et al.* (1993) *Nature* 362:747-749); Ogawa *et al.* (1993b) *Nature* 362:749-751; Rainier *et al.* (1995) *Cancer*  
30 *Res.* 55:1836-1838; Zhan *et al.* (1994) *J. Clin. Invest.* 94:445-448; Suzuki *et al.* (1994) *Nat. Genet.* 6:332-333). As expected, untreated tumor cells, as well as control tumor cells

treated with PBS, showed approximately equal expression of the two alleles of IGF2 (Fig. 1A), indicating LOI. In contrast, treatment with increasing doses of 5-azaCdR led to unequal expression of the two IGF2 alleles, with preferential expression of the allele lacking the Apa I polymorphic site (Fig. 1A). Although preferential expression of one allele was partial and not absolute, five repetitions of these experiments always gave the same results, with the predominantly expressed allele always represented by the upper band. Thus, the switch to predominant expression of one allele was not a random effect of 5-azaCdR, but was specific to one allele. Consistent switching by 5-azaCdR from equal biallelic expression to preferential expression of a single parental allele was also observed with a second cancer cell line. Thus, treatment of CQLO-205 colorectal carcinoma cells also showed a switch from biallelic to predominately monoallelic expression of IGF2, and the same allele was preferentially expressed in repeated experiments (data not shown). Thus, 5-azaCdR partially abrogated the pattern of equal biallelic expression of IGF2 in tumor cells with LOI.

We next examined the effect of 5-aza-CdR treatment on overall expression of H19. As we and others had found earlier, LOI of IGF2 is linked in Wilms tumors to down regulation of H19 (Steenman *et al.* (1994) *Nature Genet.* 7:433-439; Moulton *et al.* (1994) *Nature Genet.* 7:440-447). Untreated JEG-3 cells, like Wilms tumors, displayed little to no expression of H19 (Fig. 1B). After treatment with 0.3  $\mu$ M or 1.0  $\mu$ M 5-azaCdR, tumor cells showed a substantial increase in H19 expression (Fig. 1B), 30- or 50-fold, respectively, as determined by PhosphorImager analysis. These changes were also reproducible in the second cancer cell line. Thus, COLO-205 cells showed virtually no expression of H19 before treatment. After treatment with 1.0  $\mu$ M 5-azaCdR, cells showed a 20-fold increase in H19 expression (data not shown). Thus, 5-azaCdR abrogated the epigenetic silencing of H19 in tumor cells with LOI.

We had also observed earlier that LOI of IGF2 in Wilms tumor is associated with biallelic expression of H19 (Rainier *et al.* (1993) *Nature* 362:747-749), despite the relatively low overall quantitative expression of H19 in the tumors. Biallelic expression of H19 is also a characteristic of JEG-3 cells (Hashimoto *et al.* (1995) *Nat. Genet.* 9:109-110). We therefore examined allele-specific expression of H19. We used an RT-PCR assay that reflects allele-specific expression (Rainier *et al.* (1993) *Nature* 362:747-749) but should not

be used to quantify total mRNA levels. 5-azaCdR treatment caused a change from biallelic expression to preferential expression of a single H19 allele. This change was even more dramatic than that seen for IGF2, with monoallelic expression of the allele containing the Alu I polymorphic site (Fig. 1C).

5 We and others showed earlier that LOI is associated with abnormal methylation of the normally unmethylated maternal H19 promoter, to a degree similar to normal methylation of the paternal allele (Steenman *et al.* (1994) *Nature Genet.* 7:433-439; Moulton *et al.* (1994) *Nature Genet.* 7:440-447; Reik *et al.* (1994) *Hum. Mol. Genet.* 3:1297-1301). In order to determine whether treatment with 5-azaCdR altered methylation  
10 of this imprint-specific region, cells were digested with Pst I and Sma I, as described (Reik *et al.* (1994) *Hum. Mol. Genet.* 3:1297-1301). Normal cells show approximately 50% methylation, since the paternal allele is methylated and the maternal allele is unmethylated (Steenman *et al.* (1994) *Nature Genet.* 7:433-439; Moulton *et al.* (1994) *Nature Genet.* 7:440-447; Reik *et al.* (1994) *Hum. Mol. Genet.* 3:1297-1301). Cells with LOI show  
15 increased methylation, reflecting abnormal methylation of the maternal allele (Steenman *et al.* (1994) *Nature Genet.* 7:433-439; Moulton *et al.* (1994) *Nature Genet.* 7:440-447; Reik *et al.* (1994) *Hum. Mol. Genet.* 3:1297-1301). As expected, control cells treated with PBS showed increased methylation of the H19 CpG island (Fig. 2A). However, after treatment with 5-azaCdR, the H19 CpG island showed a pattern of methylation approaching 50%  
20 (Fig. 2A). Like the changes in IGF2 imprinting after 5-azaCdR treatment, these changes in DNA methylation were also reproducible in the second cancer cell line, COLO-205 (data not shown).

Finally, we asked whether the effects of 5-azaCdR were specific to a single allele, as would be expected if these changes were related to changes in genomic imprinting, and  
25 not a random effect of 5-azaCdR on both methylated alleles. We PCR-amplified a region of H19 containing a Hpa II site that shows imprint-specific methylation, as well as an Alu I polymorphism that distinguishes the two alleles of the gene. By digesting genomic DNA with Hpa II prior to PCR, we could determine which alleles were methylated before and after treatment of cells with 5-azaCdR. This analysis showed, as expected, that both alleles  
30 were methylated in the absence of 5-azaCdR (Fig. 2B). However, after treatment with 5-azaCdR, the allele lacking the Alu I polymorphic site was predominantly amplified (Fig.

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2B). Thus, the non-Alu I-containing allele remained methylated, while the Alu I-containing allele ~ became unmethylated after 5-azaCdR treatment. Thus, one allele was preferentially affected by 5-azaCdR. Furthermore, the change in IGF2 methylation was not affected by 5-azaCdR (data not shown), again indicating a specific effect of 5-azaCdR on these cells.

5 In summary, 5-azaCdR restored a pattern of normal genomic imprinting to tumor cells with LOI by each of the following criteria: equal biallelic expression of IGF2 switched to preferential expression of the same allele in five repeated experiments; epigenetic silencing of H19 was reversed; biallelic expression of H19 switched to monoallelic expression of the same allele in all experiments; and hypermethylation of the  
10 H19 promoter switched to demethylation of one allele but not the other. These effects on tumor cells with LOI were not random but were specific in each instance to one allele, and they were reproducible both within and between the cell lines. We have shown that 5-azaCdR switched IGF2 from approximately equal expression of maternal and paternal alleles to predominant expression of a specific parental allele in repeated experiments, and  
15 caused a switch from biallelic to monoallelic expression of H19 as well as quantitative activation of H19 expression. These effects were not a random response to 5-azaCdR, or else tumor cells would have continued to show equal expression of both alleles of IGF2 and H19. Consistent with the nonrandom effect of 5-azaCdR in these experiment, the loss of DNA methylation of the H19 promoter was specific to one allele. Thus, 5-azaCdR  
20 treatment confers a normal pattern of genomic imprinting on tumor cells with LOI. This pattern was complete for H19 and partial for IGF2, since in the latter case both alleles were expressed, although the same allele was preferentially expressed in repeated experiments.

These results have three important implications. First, they indicate that abnormal  
25 imprinting in tumor cells can be modified pharmacologically. These results are unexpected since previous work with 5-azaCdR would lead one of ordinary skill in the art to conclude that this drug would simply produce hypomethylation of both chromosomes. For example, Eversole-Cire *et al* previously showed that 5-azaCdR treatment of mouse cells with maternal disomy for chromosome 7 causes epigenetic activation of the normally silent  
30 maternal IGF2 gene on that chromosome (Eversole-Cire *et al.* (1993) *Mol. Cell. Biol.* 13:4928-4938). Similarly, Hu *et. al* recently found that treatment of normal human brain

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cells with 5-azaCdR switched IGF2 from a pattern of preferential expression of one allele to equal biallelic expression (Hu *et al.* (1993) *J. Biol. Chem.* 271:18253-18262). In contrast, in the experiments described here using tumor cells with LOI, the pattern of allele-specific expression of IGF2 unexpectedly switched from equal biallelic expression to predominant expression of a single allele. Thus, the results described here are opposite to that taught in the prior art.

Second, these experiments show that some imprint-specific information must still be present in tumor cells with LOI. These results unexpectedly show that the methylation of the H19 promoter affecting expression is independent of the imprint; and, that the imprint is intact but masked by abnormal methylation. Third, this work may provide a novel experimental strategy toward understanding the molecular basis of abnormal imprinting in cancer, since these experiments generate isogenic cell lines that differ in their imprinting of IGF2 and H19. These cell lines can then be tested for alterations at additional target sites, such as p57<sup>KIP2</sup>, which we have also found to be imprinted (Matsuoka *et al.* (1996) *Proc. Natl. Acad. Sci. USA* 93:3026-3030), or for alterations in transacting factors that may have been activated by 5-azaCdR to mediate these effects. One can use the cells or experiments like those described herein to discover other pharmacologic agents that might influence genomic imprinting.

While the results with 5-azaCdR represent a strategy for drug development, this idea is not limited to that particular compound. Additional drugs have been tested to determine whether or not they show alteration in imprinting. Drugs that show at least some alteration in imprinting and/or cytotoxic effects include lankacidin, benzenamine, blue platinum uracil, cyclohexane acetic acid, methyl 13-hydroxy-15-oxo-kaurenoate, and others. Even drugs which do not show an *in vivo* effect on imprinting may show such an effect if the drugs are modified biochemically to enhance bioavailability, drug delivery, stability, etc.



**Example 2: Determination of the propensity of an individual to develop cancer or a malignant growth.**

**Materials and Methods**

DNA and RNA was prepared from tissues from colon cancer patients and normal controls as follows. Colon cancers, normal mucosa from the same patients and normal mucosa from patients who do not have colon cancer but whose colons were removed for other reasons, such as diverticulitis. DNA was extracted by pulverizing the tissue in liquid nitrogen, followed by resuspending in 5ml of TE9 [500mM Tris, 20mM EDTA, 10mM NaCl, pH 9.0]. Samples were incubated overnight at 55°C after addition of proteinase K to 100µg/ml and SDS to a 1% final concentration. Samples were extracted twice with phenol-chloroform, once with chloroform, and DNA in the samples were ethanol precipitated, washed and resuspended in TE [3mM Tris, 0.2mM EDTA, pH 7.5]. RNA was prepared using the MicroFastTrack mRNA isolation kit from Invitrogen.

**Results**

8 informative colorectal cancer were identified with LOI of IGF2. Examination of matched normal tissue revealed that 6 of these 8 patients had LOI of the normal colon mucosal cells. In contrast, of 16 informative patients without colorectal cancer, only 3 of these patients had LOI of the normal mucosa. It is predicted that these patients may have been at risk of developing cancer. In tabular form the results are as shown below.

	LOI in normal mucosa	No LOI (i.e., normal imprinting) in normal mucosa
Colorectal cancer with LOI in the cancer tissue	13	3
Noncancer patients	2	6

These data are highly significant statistically ( $p=0.013$ ).

Thus, LOI in normal mucosa is present where there is cancer with LOI, at a significantly higher rate than where there is not cancer. It is predicted that in such

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"normal" cells with LOI, the LOI would be as reversible by the approach described herein as are cancer cells with LOI. The approach thus, finds use as a chemoprevention strategy for colon cancer with LOI. Likewise, the methods find use in other cancer or tumorigenic growth or other diseases involving LOI.

5

**Example 3: Identification of new lead compounds to restore a normal pattern of imprinting using reference database.**

Based on the results showing that 5-aza-2'-deoxycytidine can restore normal imprinting to tumor cells with LOI, an analysis was performed using the drug discovery  
10 program described in Weinstein *et al.* (1997) *Science* 275:343-349, herein incorporated by reference. The program was used specifically looking for clustered correlations, i.e., compounds that showed the greatest degree of correlation of biological activity similar to 5-  
aza-2'-deoxycytidine against the 60 reference cell lines. The highest correlations were as follows: 127716, 619003, 626883, 624646, 145118, 1850512, 626884, 626886, 622810,  
15 176889, 622616, 620358, 619948, 622461, 624645, 15309, 638737, 294734, 112152, 153174 204816, 79037, 628082, 620319 (NCI reference numbers). These are considered lead compounds to determine whether they will restore normal imprinting or could be chemically modified to do so.

As a first test of this approach, the in vitro effect of 12 of these compounds were  
20 analyzed with saline as a negative control.

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	Compound/ NCI reference number	effective concentration	name	Allele ratio 1.0=complete LOI	Partial or Complete Reversal of LOI
5	saline			1.0	-
	15309	$10^{-3}$ M	phlorone	1.3	+
	112152	$10^{-3}$ M	plumericine	1.2	+
	145118	$10^{-4}$ M	lankacyclin	1.8	++
	153174	$10^{-4}$ M	cis-CCNU	1.6	++
10	176889	$10^{-4}$ M	benzenamine	3.7	+++
	180512	$10^{-2}$ M	Blue Platinum uracil	45.0	+++++
	204816	$10^{-4}$ M	cyclohexane acetic acid	2.8	+++
	619948	$10^{-4}$ M	No formal name	1.8	++
	620358	$10^{-4}$ M	Methyl-13- hydroxy-15- oxo- kaurenoate	2.6	+++
15	624645	$10^{-4}$ M	No formal name	1.8	++
	624646	$10^{-4}$ M	No formal name	2.2	+++

Lead compounds identified in this manner did reverse LOI. Once compounds have been identified, they may be designed for maximum effect or stability. In this manner many new compounds can be developed based on the compounds identified in the above manner. Such modified compounds can be used individually in the same database to identify a second tier of lead compounds. This cycle of modification and selection can be repeated until a compound or agent of choice is obtained.

The compounds were additionally tested for their in vivo cytotoxic effects on cancer cells, using a hollow fiber assay as described in Hollingshead *et al.* (1995) *Life Sci.*

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57:131-141, herein incorporated by reference. Of these compounds, the following scored above the threshold for referral for xenograft testing, i.e., a total effectiveness score >20 or an in vivo cell kill effect seen directly: 15309, 112152, 127716 (5-aza-deoxy-cytidine, i.e., in vivo effect similar to in vitro effect), 204816, 620358, 624646. Thus, several new potential effective chemotherapeutics were identified for tumors and/or doses not taught in the literature.

A different use of the NCI database was performed by identifying cells lines in the NCI set that have LOI and those that do not. For example, see the table below:

10	<u>Cells having LOI, (Group A)</u>	<u>Cells that do not have LOI, (Group B)</u>
	SK-MEL-S melanoma	M14
	CAKI-1 renal cell cancer	OVCAR-3
	786-0 renal cell cancer	HCT-15
	HT29 colon cancer	HOP-92
15	PC-3 prostate cancer	MCF-7
	SN12C renal cancer	SK-MEL-2
	MDA-MB-435 breast cancer	MALME-307
	COLO 205 colon cancer	HCT-116
		LOX-1MV1
20		SNB-75
		SF-268
		RXF-393
		A498

25 Compounds were identified that are effective agents against Group A but not Group B. Such compounds are lead compounds to be tested for efficacy and ability to restore normal imprinting to tumors of Group A. Such compounds might have general efficacy against: 1) types of tumors that commonly show LOI; 2) individual tumors from patients with LOI for tailor-made chemotherapy; and, 3) chemoprevention in tissues with LOI.

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	Predicted concentration	NCS #	Name
	10 <sup>-4</sup> M	72055-C	sulfonium
	10 <sup>-4</sup> M	645822-0	
5	10 <sup>-4</sup> M	682841	
	10 <sup>-4</sup> M	666860	euphornin D
	10 <sup>-4</sup> M	282880-7	octadecylphosphoryl choline
	10 <sup>-4</sup> M	622688-S	
	10 <sup>-4</sup> M	694536-0	
10	10 <sup>-4</sup> M	252940-S	gndimacrin
	10 <sup>-4</sup> M	645835-C	
	10 <sup>-4</sup> M	670561-F	
	10 <sup>-4</sup> M	697032-A	
	10 <sup>-2</sup> M	200692-A	aspiculamycin HCL
15	10 <sup>-4</sup> M	665350-P	

Note: Many compounds are unnamed because they are natural derivatives or as they have been placed into the database but never tested on humans. (Where compounds are identified that have not been tested on humans, methods are available in the art for such methods of testing.)

20

All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

25

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

30

Many modifications and other embodiments of the invention will come to mind in one skilled in the art to which this invention pertains having the benefit of the teachings

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presented in the foregoing descriptions and the associated drawings. Therefore, it is to be understood that the invention is not to be limited to the specific embodiments disclosed.

Although specific terms are employed, they are used in a generic and descriptive sense only and not for purposes of limitation, and that modifications and embodiments are

5 intended to be included within the scope of the appended claims.

CLAIMS:

What is claimed is:

1. A method for restoring normal imprinting in a eukaryotic cell, said method  
5 comprising contacting said cell with an effective amount of a pharmacological agent that  
influences genomic imprinting wherein said effective amount is sufficient to targets cells  
exhibiting abnormal imprinting but does not produce general cytotoxicity.
2. The method of claim 1 wherein said eukaryotic cell is a mammalian cell.  
10
3. The method of claim 1 wherein said pharmacological agent is selected from  
the group consisting of inhibitors of DNA methylation, histone deacetylation,  
topoisomerase II, and DNA synthesis.
- 15 4. The method of claim 3 wherein said pharmacological agent is selected from  
the group consisting of 5-aza-2'deoxyctodine, Trichostatin A, lankacidin, benzenamine,  
cyclohexane acetic acid, blue platinum uracil, methyl 13-hydroxy-15-oxo-kaurenoate,  
sulfonium, euphornin D, octadecylphosphoryl choline, gnidimacrin, and aspiculamycin  
HCL.  
20
5. The method of claim 2 wherein said contacting comprises administering an  
effective amount of a pharmaceutical composition to a mammal in need thereof.
6. The method of claim 5 wherein said administration is systemic  
25 administration.
7. The method of claim 5 wherein said pharmacological agent is selected from  
the group consisting of inhibitors of DNA methylation and histone deacetylation.
- 30 8. The method of claim 7 wherein said pharmacological agent is selected from  
the group consisting of 5-aza-2'deoxyctodine, Trichostatin A, lankacidin, benzenamine,

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cyclohexane acetic acid, blue platinum uracil, methyl 13-hydroxy-15-oxo-kaurenoate, sulfonium, euphornin D, octadecylphosphoryl choline, gnidimacrin, and aspiculamycin HCL.

5           9.     A method for treating or preventing a disease involving abnormal imprinting, said method comprising administering an effective amount of a pharmacological agent that is involved in genomic imprinting to a mammal in need thereof.

10           10.    The method of claim 9 wherein said disease is selected from the group consisting of Wilm's tumor, lung cancer, paraganglioma tumor, prostate cancer, breast cancer, colon cancer, bladder cancer, leukemia, ovarian cancer, kidney cancer, and retinoblastoma.

15           11.    The method of claim 9 wherein said disease is malignant growth of a tissue or organ.

            12.    The method of claim 9 wherein said pharmacological agent is selected from the group consisting of inhibitors of DNA methylation and histone deacetylation.

20           13.    The method of claim 12 wherein said pharmacological agent is selected from the group consisting of 5-aza-2'deoxyctodine, Trichostatin A, lankacidin, benzenamine, cyclohexane acetic acid, blue platinum uracil, methyl 13-hydroxy-15-oxo-kaurenoate, sulfonium, euphornin D, octadecylphosphoryl choline, gnidimacrin, and aspiculamycin HCL.

25

            14.    A method for diagnosing the propensity of a mammal to develop cancer in a target organ or tissue, said method comprising determining the imprinting pattern of cells from said organ or tissue.



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15. The method of claim 14 wherein said organ or tissue is selected from the group consisting of colon, breast, prostate, liver, lung, bladder, kidney, ovary, and testicular.

5 16. A method to identify pharmacological agents involved in genomic imprinting, said method comprising administering a pharmacological agent to a cell and determining the imprinting pattern of said cell.

10 17. A pharmaceutical composition comprising a pharmacological agent involved in imprinting, said composition comprising an effective amount of a pharmacological agent that influences genomic imprinting wherein said effective amount is sufficient to targets cells exhibiting abnormal imprinting but does not produce general cytotoxicity.

15 18. The method of claim 17 wherein said pharmacological agent is selected from the group consisting of inhibitors of DNA methylation and histone deacetylation.

20 19. The method of claim 18 wherein said pharmacological agent is selected from the group consisting of 5-aza-2'deoxycytodine, Trichostatin A, lankacidin, benzenamine, cyclohexane acetic acid, blue platinum uracil, methyl 13-hydroxy-15-oxo-kaurenoate, sulfonium, euphornin D, octadecylphosphoryl choline, gnidimacrin, and aspiculamycin HCL.

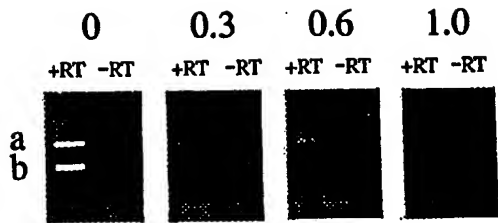


FIG. 1A.

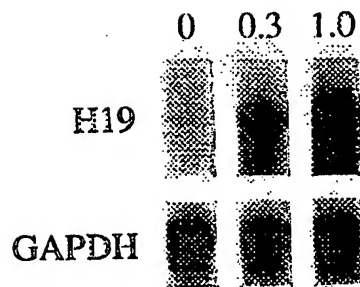


FIG. 1B.

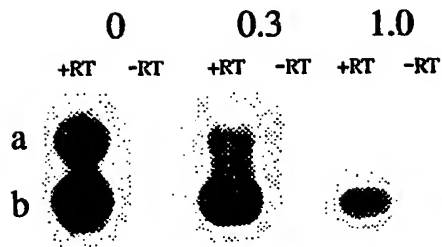


FIG. 1C.

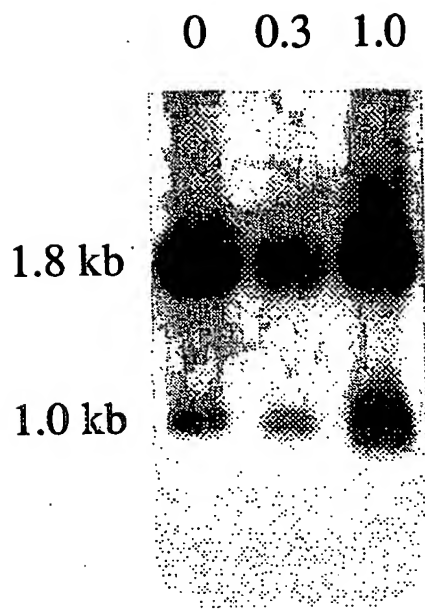


FIG. 2A.

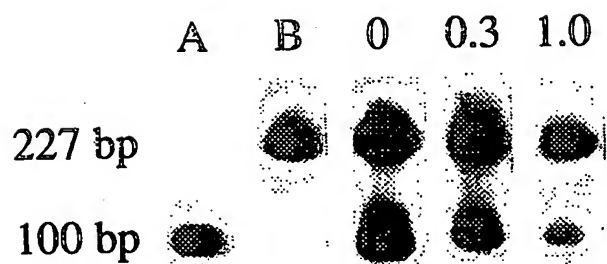


FIG. 2B.

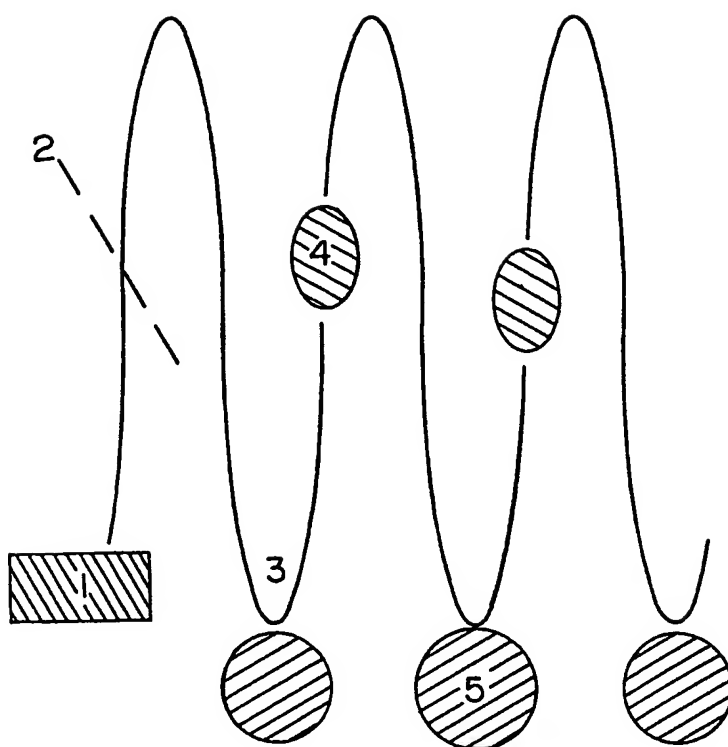


FIG. 3.